Activation of NF-κB induced by H$_2$O$_2$ and TNF-α and its effects on ICAM-1 expression in endothelial cells

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True, Andrea L., Arshad Rahman, and Asrar B. Malik. Activation of NF-κB induced by H$_2$O$_2$ and TNF-α and its effects on ICAM-1 expression in endothelial cells. Am J Physiol Lung Cell Mol Physiol 279: L302–L311, 2000.—Reactive oxygen species have been proposed to signal the activation of the transcription factor nuclear factor (NF)-κB in response to tumor necrosis factor (TNF)-α challenge. In the present study, we investigated the effects of H$_2$O$_2$ and TNF-α in mediating activation of NF-κB and transcription of the intercellular adhesion molecule (ICAM)-1 gene. Northern blot analysis showed that TNF-α exposure of human dermal microvascular endothelial cells (HMEC-1) induced marked increases in ICAM-1 mRNA and cell surface protein expression. In contrast, H$_2$O$_2$ added at subcytolytic concentrations failed to activate ICAM-1 expression. Challenge with H$_2$O$_2$ also failed to induce NF-κB-driven reporter gene expression in the transduced HMEC-1 cells, whereas TNF-α increased the NF-κB-driven gene expression ~10-fold. Gel supershift assay revealed the presence of p65 (Rel A), p50, and c-Rel in both H$_2$O$_2$- and TNF-α-induced NF-κB complexes bound to the ICAM-1 promoter, with the binding of the p65 subunit being the most prominent. In vivo phosphorylation studies, however, showed that TNF-α exposure induced marked phosphorylation of NF-κB p65 in HMEC-1 cells, whereas H$_2$O$_2$ had no effect. These results suggest that reactive oxygen species generation in endothelial cells mediates the binding of NF-κB to nuclear DNA, whereas TNF-α generates additional signals that induce phosphorylation of the bound NF-κB p65 and confer transcriptional competency to NF-κB.

Reactive Oxygen Species (ROS) play a critical role in the mechanism of inflammation associated with sepsis and acute respiratory distress syndrome (17). ROS can function as second messengers in the signal transduction pathways activated by the proinflammatory cytokines interleukin-1β and tumor necrosis factor (TNF)-α (28, 31). These cytokines regulate the redox-sensitive transcription factor nuclear factor (NF)-κB, which participates in a variety of immune, inflammatory, and acute-phase responses (4, 6). NF-κB/Rel transcription factors are composed of five distinct DNA binding subunits: NF-κB1 (p50), NF-κB2 (p100/p52), Rel A (p65), Rel B, and c-Rel (23). Of these, p65, Rel B, and c-Rel possess both transactivation and DNA binding domains, whereas p50 and p52 lack the transactivation domain and serve primarily as DNA binding subunits (36). The different family members can associate in homodimer or heterodimer combinations through the highly conserved NH$_2$-terminal NF-κB/Rel/Dorsal or Rel homology domain (16). NF-κB dimers are most commonly composed of p65 and p50 or p52 subunits (3, 39). Inactive NF-κB dimers are sequestered in the cytosol in association with inhibitory molecules of the IkB family. Stimulation of cells with TNF-α results in phosphorylation of IkB-α on serine-32 and -36 or of IkB-β on serine-19 and -23 by IkB kinases α and β (13, 46). This targets IkB-α and IkB-β for polyubiquitination and proteasome-mediated degradation (11, 43). Release from IkB unmasks the nuclear localization signal of NF-κB and thus mediates its translocation to the nucleus (7).

Although phosphorylation of IkB is a critical regulatory step in the activation of NF-κB, studies (8, 36) suggested that phosphorylation of NF-κB p65 after its release from IkB may enhance its transactivation potential. Thus phosphorylation of NF-κB may be an additional mechanism regulating the expression of NF-κB-dependent genes. In the present study, we addressed the activation of such a gene, intercellular adhesion molecule-1 (ICAM-1), an inducible cell surface glycoprotein belonging to the immunoglobulin supergene family (42). Interaction of ICAM-1 with its counterreceptors (leukocyte β$_2$-integrins CD11a/CD18 and CD11b/CD18) is a requirement for the recruitment and extravasation of leukocytes to sites of tissue injury and infection (40, 41). ICAM-1 mRNA and cell surface expression are upregulated in response to a variety of inflammatory mediators including TNF-α (18, 22, 33).

Rahman and colleagues (31, 32) have shown that TNF-α induces ROS generation in endothelial cells through a protein kinase (PK) C-dependent mechanism and that this event is critical in signaling the activation of NF-κB and the genes encoding ICAM-1 and E-selectin. Studies (35, 37) have also shown that H$_2$O$_2$ mediates TNF-α-induced NF-κB activation; however, there are reports (10, 21) that ROS may not...
signal the activation of NF-κB and NF-κB-dependent gene expression. A study (20) has shown that H₂O₂ induced the expression of ICAM-1 mRNA in a human dermal microvessel endothelial cell (HMEC-1) line; in other studies, H₂O₂ failed to induce DNA binding activities of the transcription factors activator protein (AP)-1 and NF-κB (21) that are known to activate ICAM-1 gene transcription in endothelial cells (18, 22, 26). In light of these observations, we compared the effects of H₂O₂ with those of TNF-α in mediating the activation of NF-κB in endothelial cells using HMEC-1 cells. We demonstrated that both H₂O₂ and TNF-α induced the DNA binding activity of NF-κB; however, the binding of NF-κB induced by H₂O₂ was transcriptionally “silent” as determined by its inability to activate the NF-κB-driven reporter gene and ICAM-1 expression. In contrast, the NF-κB binding activity induced by TNF-α was transcriptionally active. These data suggest that ROS production signals nuclear NF-κB DNA binding activity; however, the bound NF-κB may remain transcriptionally inactive. Thus TNF-α activates additional signaling pathway(s) that induce phosphorylation of NF-κB p65 and confer transcriptional competency to the DNA-bound NF-κB.

METHODS

Cell culture. Human dermal microvascular endothelial cells (HMEC-1) were maintained in culture in MCDB-131 medium (GIBCO BRL, Life Technologies, Gaithersburg, MD) supplemented with 5% fetal bovine serum (FBS), 10 ng/ml of human epidermal growth factor (Sigma, St. Louis, MO), 1 μg/ml of hydrocortisone, 5 mg/ml of l-glutamine, and antibiotics (penicillin-streptomycin) (“complete” MCDB-131). HMEC-1 cells, an immortalized endothelial cell line transformed by the SV40 large T antigen, retain the endothelial phenotype and functional characteristics; they express and secrete von Willebrand factor, take up acetylated low-density lipoprotein, form tubes when grown in Matrigel, and express CD31, CD35, ICAM-1 (CD54), and CD44 (1). HMEC-1 cells were passaged in uncoated culture dishes until they reached confluence. The cells between passages 17 and 21 were used in these studies, and all cell studies were carried out under the same conditions. Confluent HMEC-1 cells were serum starved for 2–4 h, washed two times with serum- and phenol red-free Dulbecco’s modified Eagle’s medium, allowed to equilibrate for 30 min, and then incubated with the indicated concentrations of H₂O₂ (Sigma) or recombinant human TNF-α (specific activity 2.3 × 10⁷ U/mg protein; Promega, Madison, WI) for all experiments unless otherwise specified.

Cell viability. Two methods were used to evaluate cell viability after H₂O₂ and TNF-α challenge. Trypan blue (Sigma) exclusion studies were carried out according to manufacturer’s suggested protocol. Confluent cells were treated with H₂O₂ or TNF-α for 2 h, washed gently with PBS two times, trypsinized, resuspended, and washed with complete MCDB-131. The cell suspension (10 μl) was mixed with 10 μl of 1× trypan blue solution, and 10 μl of the resulting mixture were loaded onto a hemocytometer. Results showed that 95–98% of the cells were viable after challenge with 100 μM H₂O₂ or 100 U/ml of TNF-α, whereas only 70–80% of the cells were viable after 1 mM H₂O₂ challenge. Reduction of tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was also used to assay cell viability (19). These results showed that HMEC-1 cells exposed to 100 μM H₂O₂ were viable after treatment for 5, 60, or 120 min, whereas cells challenged with 1 mM H₂O₂ were not (Lum H, personal communication).

Intracellular oxidant generation. Oxidant generation in HMEC-1 cells was measured as previously described (32). Briefly, confluent HMEC-1 cells were challenged with TNF-α or H₂O₂ for 15 min in serum-free phenol red-free DMEM, and all subsequent steps were conducted in the dark. After challenge, the cells were washed two times with MCDB-131 (2% FBS) and loaded for 20 min with 1 μM 5-(and-6)-carboxy-2’,7’-dichlorodihydrofluorescein diacetate bis(acetoxymethyl ester) (C-DCHF-DA; Molecular Probes, Eugene, OR). The dye solution was freshly prepared in prewarmed MCDB-131 (2% FBS) for each sample, and the samples were independently stained so that they were exposed to dye for the same amount of time. After dye loading at 37°C, the cells were rinsed two times with MCDB-131 (2% FBS) containing no dye, and the cultures were viewed with a fluorescence microscope and photographed. Quantitative fluorescence was imaged with a Nikon Diaphot 200 microscope (Nikon, Garden City, NY) and ImagePro Plus software (Media Cybernetics, Silver Spring, MD).

Northern analysis. Total RNA was isolated from HMEC-1 cells with QIAGEN RNeasy columns after homogenization through QIAshredder columns (QIAGEN, Chatsworth, CA). Quantification and purity of RNA were assessed by the ratio of absorbance at 260 nm to that at 280 nm. An aliquot of RNA (10–20 μg) was analyzed for ICAM-1 mRNA expression as previously described (30). Autoradiography was performed with an intensifying screen at −70°C for 12–24 h.

Flow cytometric analysis. HMEC-1 monolayers on six-well tissue culture dishes were left untreated or were stimulated with H₂O₂ or TNF-α as indicated, washed two times with cold PBS (Ca²⁺ and Mg²⁺ free), removed by trypsinization, and incubated in 20% horse serum for 30 min at 4°C to block nonspecific binding. Samples were pelleted at 4°C and washed two times with cold 3% horse serum in PBS. The cells were incubated in the same wash buffer with the anti-human ICAM-1 monoclonal antibody RR1/1 (5–10 μg/ml) (provided by Dr. Robert Rothlein, Boehringer Ingelheim, Ridgefield, CT) (14), washed two times, and incubated with a FITC-conjugated goat anti-mouse secondary antibody (10–100 μg/ml; Sigma). After two washes and fixation with 2% paraformaldehyde while being vortexed, the samples were analyzed by flow cytometry in a FACScan cytofluorometer (Beckton Dickinson, Mountain View, CA), with mean fluorescence intensity gated above that of the secondary antibody alone. When purified mouse IgG (1 mg/ml) was used in place of RR1/1, no significant increase in fluorescence was observed.

Reporter gene construct, endothelial cell transfection, and luciferase assay. The NF-κB-luciferase plasmid (pNF-κB-Luc) containing five copies of the consensus NF-κB binding site was purchased from Stratagene (La Jolla, CA). HMEC-1 cells were plated on six-well tissue culture dishes and transfected the following day at ~70–80% confluence according to the DEAE-dextran method with slight modifications (12). Briefly, 2 μg of DNA were mixed with 0.15 mg/ml of DEAE-dextran and 0.1 mM chloroquine in serum- and antibiotic-free MCDB-131 for 1.5 h followed by a 30-s shock with 10% dimethyl sulfoxide in PBS. The cells were then washed two times with complete MCDB-131 containing 10% FBS and grown to confluence. To determine transfection efficiency, HMEC-1 cells were transfected with the plasmid pGreen Lantern-1, which expresses green fluorescence protein (GIBCO BRL), and the transfected cells were then subjected...
to flow cytometry to determine green fluorescence protein expression. Using the above protocol, we showed that transfection efficiencies were ~50%, with ~5% variability in a given sample. After transfection, the cells were exposed to H2O2 or TNF-α at the indicated concentrations and time periods. Cell extracts were prepared, and luciferase activity was determined with a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA) with the Promega Biotech luciferase reporter assay system. Values are expressed as relative light units per microgram of protein extract, and the protein content was determined with the Bio-Rad (Hercules, CA) protein determination kit.

Nuclear protein isolation. Cells were washed three times with ice-cold Tris-buffered saline (TBS) and resuspended in 400 μl of buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μg/ml of leupeptin, and 5 μg/ml of aprotinin]. After 15 min, Nonidet P-40 (NP-40) was added to a final concentration of 0.6%. Nuclei were pelleted and suspended in 50 μl of buffer B [20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 5 μg/ml of leupeptin, and 5 μg/ml of aprotinin]. After 30 min of agitation at 4°C, the lysates were centrifuged, and the supernatants containing the nuclear proteins were transferred to fresh vials in 15-μl aliquots and stored at −70°C until used.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was performed as previously described (31). Briefly, nuclear extract (10 μg) was incubated with 1 μg of poly(dI-dC) in binding buffer [10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM DTT, and 10% glycerol; 20 μl final volume] for 15 min at room temperature. End-labeled, double-stranded oligonucleotides (30,000 counts/min each) were then added in the absence and presence of a 25- to 100-fold molar excess of cold wild-type (ICAM-1NF-κB) or mutant (mut-ICAM-1NF-κB) competitor and incubated for 15 min at room temperature. In antibody supershift experiments, nuclear extracts were incubated for 15 min at room temperature with polyclonal rabbit antibody to various NF-κB proteins (2 μg/20 μl; Santa Cruz Biotechnology, Santa Cruz, CA) before incubation with the labeled probe for another 15 min at room temperature. The DNA-protein complexes were resolved by 5–6% native polyacrylamide gel electrophoresis (PAGE) in low ionic strength buffer (0.25× Tris-borate-EDTA). Oligonucleotides used for the gel shift analysis were 5’-AGGCTGGAATTTCCGGAGCT-3’ for ICAM-1NF-κB and 5’-AGGCTTCCAAATTCGGAGCTG-3’ for mut-ICAM-1NF-κB. The oligonucleotide designated as ICAM-1NF-κB represents the 21-bp sequence of ICAM-1 promoter encompassing the downstream NF-κB binding site located 223 bp upstream from the transcription initiation site (18, 22). The oligonucleotide mutant-ICAM-1NF-κB is similar to ICAM-1NF-κB except that it has 2-bp mutations in the NF-κB binding site. NF-κB sequence motifs within the oligonucleotides are underlined and mutations are shown in lowercase. In separate experiments, nuclear proteins were incubated with the oligonucleotide Ig-kB (5’-AGGCTGGAAGCCTTCCGGAGC-3’), which contains the consensus NF-κB binding site present in the Ig gene and pNF-κB-Luc construct.

In vivo labeling of cells and immunoprecipitation. In vivo labeling of cells and immunoprecipitation were carried out as described by Olliver et al. (27) with slight modifications. Briefly, confluent monolayers of HMEC-1 cells on six-well tissue culture dishes were washed twice with phosphate-free medium and incubated for 2 h before being loaded with 200–500 μCi [32P]orthophosphate/ml for 3–4 h, followed by stimulation with TNF-α or H2O2. Cells were needle lysed in 500 μl of cold radioimmunoprecipitation assay buffer [1% Triton X, 1% deoxycholate, 150 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EGTA, 1 mM PMSF, 1 mM Na3VO4, 0.1% SDS, 5 μg/ml of aprotinin, and 1 nM calyculin A]. NF-κB p65 was recovered by immunoprecipitation (after preclearance with 50 μl of protein A agarose beads) with an anti-p65 antibody (1 μg, 4–18 h at 4°C; Santa Cruz Biotechnology) and protein A agarose beads (2 h at 4°C; Boehringer Mannheim, Indianapolis, IN). The precipitated proteins were washed three times, including one high salt wash (radioimmunoprecipitation assay with 500 mM NaCl), and were then pelleted. The antibody-p65-bead complexes were boilded in sample buffer containing 25 mM DTT for 5 min and spun, and the supernatants were separated on 10–12.5% SDS-polyacrylamide gels for 4.5 h (20 mA). Comassie blue staining of the gels revealed the presence of a single band corresponding to a molecular mass of 65 kDa. The gels were then either dried and visualized by autoradiography or transferred to polyvinylidene difluoride membranes for Western blotting and confirmation of NF-κB p65.

Western blot analysis. The immunoprecipitated NF-κB p65 samples were subjected to SDS-PAGE (10%) as described in In vivo labeling of cells and immunoprecipitation and then transferred to polyvinylidene difluoride membranes (Bio-Rad). The membranes were blocked for 1 h with 5% (wt/vol) nonfat dry milk solution in 10 mM Tris base, 150 mM NaCl, and 0.05% Tween 20 before the membranes were incubated for 1 h with rabbit polyclonal anti-human NF-κB p65 antibodies (Santa Cruz Biotechnology) diluted 1:1,000. The membranes were washed three times with TBS-Tween 20 and incubated for another hour with goat anti-rabbit horseradish peroxidase-linked IgG (Amersham, Arlington Heights, IL) diluted 1:5,000. After the washes, antibody-labeled proteins were detected by the enhanced chemiluminescence method (Amersham) according to manufacturer’s recommendations.

RESULTS

H2O2 failed to mimic TNF-α-induced expression of ICAM-1 in HMEC-1 cells. We compared the effects of H2O2 with those of TNF-α on NF-κB-dependent ICAM-1 gene expression. Northern blot analysis showed that H2O2 failed to activate ICAM-1 transcription in HMEC-1 cells at all concentrations studied, whereas TNF-α induced robust ICAM-1 mRNA expression (Fig. 1). HMEC-1 cells were subjected to fluorescence-activated cell-sorting analysis with the anti-ICAM-1 monoclonal antibody RR1/1 (14) to determine whether H2O2 altered the cell surface expression of ICAM-1 independent of increased mRNA expression. As shown in Fig. 2, H2O2 failed to induce ICAM-1 cell surface expression. In contrast, TNF-α induced a marked increase in ICAM-1 cell surface expression within 2 h, which increased further after 18 h of exposure (Fig. 2).

TNF-α and H2O2 challenges induced similar intracellular oxidant generation. To determine whether differences in ICAM-1 induction by H2O2 and TNF-α could be ascribed to differences in the oxidant-generating capacity of the mediators, confluent HMEC-1 cells were challenged with TNF-α (100 U/ml) or H2O2 (subcytolytic concentrations ranging from 10 to 500 μM) for 15 min and loaded with the oxidant-sensitive...
dye C-DCHF-DA as described in METHODS. After incorporation and subsequent cleavage by cellular esterases, C-DCHF-DA was trapped in cells and converted to the fluorescent species carboxydichlorofluorescein after oxidation. Results showed that 100 U/ml of TNF-\(\alpha\) and 100 and 500 \(\mu M\) \(H_2O_2\) both induced comparable C-DCHF-DA fluorescence compared with that in control cells, whereas the fluorescence activated by 10 \(\mu M\) \(H_2O_2\) was similar to the control values (Fig. 3A). Figure 3B shows the quantification of the relative fluorescence intensity of each field shown in Fig. 3A.

Total relative fluorescence for each image was divided into three classes of brightness where class 1 represents the area of each cell with lowest brightness intensity and class 3 represents the area of each cell with highest brightness intensity. Control cells exhibited fluorescence in brightness class 1, and treatment with 100 and 500 \(\mu M\) \(H_2O_2\) or 100 U/ml of TNF-\(\alpha\) caused a shift to a higher brightness, with maximum fluorescence occurring in brightness class 2 (Fig. 3B). These results indicate that differences in ICAM-1 expression are not secondary to a differential oxidant-generating capacity of the two mediators.

\(H_2O_2\) failed to mimic TNF-\(\alpha\)-induced transcriptional activity of NF-\(\kappa B\). We next evaluated the ability of \(H_2O_2\) to induce transcriptional activation of NF-\(\kappa B\) because oxidants generated in response to TNF-\(\alpha\) are critical in signaling NF-\(\kappa B\) activation and ICAM-1 (31). We transiently transfected HMEC-1 cells with the plasmid pNF-\(\kappa B\)-Luc containing five copies of the consensus NF-\(\kappa B\) site linked to a minimal adenovirus E1B promoter-luciferase reporter gene. NF-\(\kappa B\)-directed luciferase activity increased ~10-fold when the transfected cells were exposed to TNF-\(\alpha\) (Fig. 4). In contrast, \(H_2O_2\) failed to induce NF-\(\kappa B\)-directed luciferase expression (Fig. 4). Because \(H_2O_2\) failed to induce transcriptional activation of pNF-\(\kappa B\)-Luc, we focused our attention on NF-\(\kappa B\) to explain the basis for the lack of ICAM-1 induction.

\(H_2O_2\) and TNF-\(\alpha\) induced NF-\(\kappa B\) DNA binding activity. Because the antioxidant actions of N-acetylcysteine and pyrrolidine dithiocarbamate prevented TNF-\(\alpha\)-induced NF-\(\kappa B\) DNA binding activity (31, 32), we determined whether the inability of \(H_2O_2\) to activate ICAM-1 transcription could be explained by the failure of NF-\(\kappa B\) to bind to the ICAM-1 promoter. We performed the EMSA using the oligonucleotide ICAM-1NF-\(\kappa B\) (21 bp), which contains the downstream NF-\(\kappa B\) sequence in the ICAM-1 promoter required for ICAM-1 expression (18, 22). Both \(H_2O_2\) and TNF-\(\alpha\) induced marked binding of NF-\(\kappa B\) to the ICAM-1 pro-
moter (Fig. 5A). These results also showed that H$_2$O$_2$ induced NF-$\kappa$B binding in a time-dependent manner, with the maximum response occurring after 30–60 min (Fig. 5B).

We next determined the specificity of NF-$\kappa$B binding activity. Competition experiments were performed in which a 25- and a 100-fold molar excess of unlabeled ICAM-1NF-$\kappa$B oligonucleotide were incubated with the labeled ICAM-1NF-$\kappa$B oligonucleotide. Excess unlabeled ICAM-1NF-$\kappa$B quenched the binding of NF-$\kappa$B to the labeled ICAM-1 promoter sequence in a concentration-dependent manner, with greater inhibition in the presence of a 100-fold molar excess of oligonucleotide (Fig. 5C, lanes 2 and 3). In contrast, incubation of labeled ICAM-1NF-$\kappa$B with excess unlabeled mutant oligonucleotide (mut-ICAM-1NF-$\kappa$B; 2-bp mutation in ICAM-1NF-$\kappa$B) had little effect on NF-$\kappa$B binding (Fig. 5C, lanes 4 and 5). The small reduction in NF-$\kappa$B binding in the presence of excess mut-ICAM-1NF-$\kappa$B oligonucleotide (Fig. 5C, lane 5) can be ascribed to the binding of NF-$\kappa$B to the mutant oligonucleotide (Fig. 5C, lane 6). Taken together, these data indicate that H$_2$O$_2$ induces the specific binding of NF-$\kappa$B to the ICAM-1 promoter in a manner similar to the binding induced by TNF-$\alpha$ (31).

Because the consensus NF-$\kappa$B sequence of pNF-$\kappa$B-Luc differs from the NF-$\kappa$B sequence present in the ICAM-1 promoter, we also performed an EMSA using an oligonucleotide that contains this sequence to determine whether H$_2$O$_2$ can induce the binding of NF-$\kappa$B to this site. As shown in Fig. 6, both H$_2$O$_2$ (lanes 2 and 3) and TNF-$\alpha$ (lane 4) caused the binding of NF-$\kappa$B to the consensus sequence. Thus the failure of H$_2$O$_2$ to induce NF-$\kappa$B-dependent reporter gene activation and ICAM-1 expression as shown above is independent of the NF-$\kappa$B DNA binding activity induced by H$_2$O$_2$.
H$_2$O$_2$- and TNF-α-induced NF-κB complexes were predominantly composed of NF-κB p65. The gel super-shift assay was carried out to determine whether differences in the H$_2$O$_2$ and TNF-α responses could be explained by differences in the NF-κB complex bound to the ICAM-1 promoter. Antibody directed against p50 (which lacks the transactivation domains), p65, or c-Rel (which contain the transactivation domains) were incubated with nuclear extracts isolated from H$_2$O$_2$- or TNF-α-treated cells before addition of the labeled ICAM-1NF-κB oligonucleotide. Antibody to p65 significantly reduced both H$_2$O$_2$ (Fig. 7A, lane 4) - and TNF-α (Fig. 7, A, lane 6, and B, lanes 6 and 7)-induced NF-κB binding to the ICAM-1 promoter. In contrast, antibodies against p50 and c-Rel had less effect on DNA binding activities (Fig. 7, A, lanes 2, 3, and 7, and B, lanes 4 and 5). Therefore, differential binding of NF-κB subunits to DNA induced by H$_2$O$_2$ and TNF-α fails to explain the observed differences in the transcriptional activation responses induced by the two mediators.

TNF-α and H$_2$O$_2$ differentially phosphorylated NF-κB p65 subunit. Because phosphorylation of NF-κB p65 may regulate transcriptional activation of NF-κB (2, 45), we determined whether alterations in the phosphorylation status of NF-κB p65 after H$_2$O$_2$ and TNF-α stimulation could explain the differential response. Cells were loaded with [$α$-32P]orthophosphate in phosphate-free medium and stimulated with H$_2$O$_2$ or TNF-α for 30 min. Immunoprecipitation of NF-κB p65 followed by SDS-PAGE showed marked phosphorylation of p65 in response to TNF-α (Fig. 8A, lane 3) but not to H$_2$O$_2$ (Fig. 8A, lane 2). Similar results were obtained after 15 min of H$_2$O$_2$ or TNF-α challenge (data not shown). To rule out the possibility that the phosphorylated proteins observed did not represent coimmunoprecipitation of other NF-κB subunits associated with p65, Western blot analysis was
performed with the anti-p65 antibody. These results indicated the selective recovery of p65 after immunoprecipitation (Fig. 8B).

**DISCUSSION**

Oxidants have been proposed to function as second messengers in the activation of transcription factors (5, 24, 28). Studies have shown that H$_2$O$_2$ induces the activation of NF-κB and the transcription of NF-κB-dependent genes in several cell types (37). Although oxidants can induce binding of NF-κB in endothelial cells (5), it remains unclear whether additional signals are involved in the transcriptional activation of NF-κB-dependent genes such as ICAM-1.

Oxidant generation induced by TNF-α in endothelial cells contributes to the mechanism of NF-κB activation and transcription of the genes encoding ICAM-1 and E-selectin (31, 32). In the present study, we determined whether H$_2$O$_2$ mimics the effects of TNF-α in mediating the activation of NF-κB and ICAM-1 expression. We demonstrated that H$_2$O$_2$ promoted the binding of the NF-κB p65 subunit to its cognate site; interestingly, the bound NF-κB remained transcriptionally “silent.” In contrast, the TNF-α-induced binding of NF-κB p65 promoted a robust NF-κB-driven reporter gene activation and ICAM-1 expression. The results suggest that the transcriptional activity of NF-κB induced by TNF-α is conferred by phosphorylation of the NF-κB p65 subunit, whereas H$_2$O$_2$ has no effect on the phosphorylation of this subunit. Thus phosphorylation of NF-κB p65 appears to be critical in mediating the transcriptional activation of NF-κB-dependent genes in endothelial cells.

H$_2$O$_2$ is a permeable oxidant that rapidly enters cells but is also rapidly degraded by intracellular anti-oxid-
The kinase responsible for p65 phosphorylation in endothelial cells is unknown. Casein kinase-II and MAPKs have been proposed to phosphorylate p65 in vitro (8, 44). PKC-ζ may also regulate phosphorylation and transcriptional activity of NF-κB p65 in endothelial cells (2) and thus may mediate ICAM-1 gene transcription after TNF-α stimulation (31). In addition, phosphorylation of NF-κB p65 by the catalytic subunit of PKA may activate NF-κB (47). NF-κB p65 phosphorylation may also be necessary for association with the coactivator p300/cAMP-responsive element binding protein (CBP), a requirement for NF-κB transactivation (15, 29). PKA-induced phosphorylation of NF-κB p65 exposed the binding site for interaction with p300/CBP (48), suggesting a mechanism by which p65 phosphorylation can induce transcriptional activation. Thus failure of phosphorylation of NF-κB p65 by one or more of these kinases could explain the inability of H2O2 to induce NF-κB gene expression in HMEC-1 cells.

Another explanation for our results is that H2O2 fails to activate transcription factors that may coordinate regulatory transcription of the ICAM-1 gene. For example, CAAT enhancer binding protein (C/EBP) was reported to cooperate with NF-κB in the mechanism of TNF-α-induced ICAM-1 expression (18). We showed that H2O2 did not induce transcriptionally active NF-κB in cells transfected with the pNF-κB-Luc construct. Because this construct is driven by NF-κB sequences and C/EBP does not bind to these sequences, it is unlikely that the failure of H2O2 to activate C/EBP can explain our observations. The role of AP-1 was also proposed in the mechanism of ICAM-1 transcription (26), although the ability of H2O2 to activate AP-1 in endothelial cells remains controversial (21). The results obtained with pNF-κB-Luc reporter construct argue that transcriptionally silent NF-κB is the more likely explanation for the absence of ICAM-1 induction by H2O2. A recent study (9) has shown that p50 can act as a negative regulator of NF-κB-dependent gene expression. Because we did not find that H2O2 preferentially induced p50 binding, it is unlikely that H2O2 prevented the transcriptional activation of NF-κB and expression of ICAM-1 by this mechanism.

The present observations differ from those in previous reports (20, 34). The cell culture conditions may have been different such that there may be differences in the redox state of cells. This could result in oxidant-induced activation of NF-κB and, thereby, ICAM-1 expression. The present results are important because they unmask a phosphorylation-dependent mechanism that may signal transcriptional activation of NF-κB after NF-κB binding to DNA.

Although H2O2 did not induce transcriptionally active NF-κB, the present results are fully consistent with our observations that TNF-α-induced ICAM-1 expression can be blocked by antioxidants (31). Because the results point to an important role for oxidants generated by TNF-α in signaling the binding of NF-κB to the promoter, it would be expected that antioxidants would prevent TNF-α-induced NF-κB binding and the resultant ICAM-1 expression as demonstrated (31).

In summary, the present results show that H2O2 induces the nuclear DNA binding activity of NF-κB and that the binding of NF-κB induced by H2O2 alone is insufficient to activate NF-κB-dependent reporter


diants. To ensure that intracellular antioxidants did not scavenge H2O2 and interfere with the response, we made studies in which HMEC-1 cells transfected with pNF-κB-Luc were exposed to freshly prepared H2O2 every 2 h for up to a 6-h period. Replenishment of H2O2 in these studies failed to induce transcriptional activity of NF-κB (data not shown); thus the present results cannot be explained on the basis of degradation of H2O2.

There may be various reasons for the failure of H2O2 to activate NF-κB-dependent transcription in HMEC-1 cells. One possibility is that H2O2 inhibits NF-κB-dependent transcription by oxidizing the critical cysteine residue required for NF-κB DNA binding activity (25). We observed that H2O2 was capable of promoting NF-κB DNA binding activity but that the bound NF-κB was transcriptionally inactive; thus under the conditions of our experiment, oxidation of NF-κB does not appear to be responsible for failure of H2O2 to induce NF-κB activity. Another possibility is that there may be differences in the redox state resulting from differences in oxidant generation. To address this question, endothelial cells were loaded with the oxidant-sensitive dye C-DCHF-DA with which we assayed oxidant generation in response to H2O2 and TNF-α. Because challenge of cells with H2O2 and TNF-α resulted in comparable dye fluorescence, any difference in the redox state cannot explain the failure of H2O2 to activate NF-κB in HMEC-1 cells. Another possibility is that H2O2 and TNF-α differentially activate the mitogen-activated protein kinase (MAPK) pathway regulating NF-κB-dependent gene expression (44). However, in other studies, we have shown that both H2O2 and TNF-α induced comparable and rapid phosphorylation of MAPK in HMEC-1 cells (True A, Lum H, Beno DW, and Malik AB, unpublished results); thus differences in activation of this pathway also cannot explain the present observations.

The question arises as to what confers transcriptional competency to the DNA-bound NF-κB after TNF-α challenge. Previous studies (22, 38) showed that overexpression of NF-κB p65 in endothelial cells transactivated the ICAM-1 and vascular cell adhesion molecule-1 promoters in a κB site-dependent manner. Phosphorylation of serine-529 in the transactivation domain 1 of NF-κB p65 in HeLa cells exposed to TNF-α may promote NF-κB transcriptional activation (45). Phosphorylation at this residue was specific in that it may promote NF-κB transcriptional activation (45).
gene activation and ICAM-1 transcription. These results suggest that the H_{2}O_{2}-induced DNA binding activity of NF-κB in the absence of phosphorylation of NF-κB p65 fails to activate ICAM-1 gene transcription. Thus TNF-α-induced phosphorylation of NF-κB p65 may be an important factor regulating expression of the NF-κB-dependent ICAM-1 gene in endothelial cells.

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